In Vitro Conversion of Leucine to Valine: Configurational Assignment of [5-^{13}C]Leucines†

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ABSTRACT: Chiral (2*S*)-[5-^{13}C]leucine was obtained from *Escherichia coli* deficient in the synthesis of acetolactate when cultures were supplemented with (*RS*)-[2-$^{13}CH_3$]acetolactate. The carbon-13 nuclear magnetic resonance spectrum showed one strong peak with a chemical shift of 21.4 ppm relative to tetramethylsilane [Sylvester, S. R., & Stevens, C. M. (1979) *Biochemistry 18*, 4529–4531]. Silver picolinate oxidation of the labeled leucine gave isovaleric acid which was then brominated at the α position to give (2*RS*)-2-bromo[3-$^{13}CH_3$]-isovaleric acid (2-bromo-3-[^{13}C]methylbutanoic acid). Aminolysis afforded (2*RS*)-[4-^{13}C]valine which was treated with D-amino acid oxidase in the presence of catalase. The final product was identified as (2*S*,3*S*)-[4-^{13}C]valine by the specificity of D-amino acid oxidase, by amino acid analysis, and by the persistence of a strong signal at δ 17.8 in the carbon-13 magnetic resonance spectrum. These results establish the absolute configuration of the biosynthetic leucine to be (2*S*,4*S*)-[5-^{13}C]leucine.

Although the principal features of the biosynthetic and degradative pathways of branched chain amino acids are well established [cf. Meister (1965)], the enzymatic mechanisms continue to be elucidated (Hill et al., 1979; Tanaka et al., 1980). In addition, recently a new pathway of leucine biosynthesis has been proposed (Poston, 1980). In all cases, characterization of the molecular intermediates has been a key in discovering the pathways, and determining absolute stereochemical configurations of the intermediates has added to the understanding of reaction mechanisms.

We have recently addressed the prochirality [see Bentley (1969) and references cited therein] of the diastereotopic β- and γ-methyl groups of valine and leucine, respectively (Sylvester & Stevens, 1979). These methyl groups are spatially nonequivalent and give rise to separate signals in the ^{13}C NMR spectrum (Tran-Dinh et al., 1974). In the case of valine, the *R* or *S* configuration of the prochiral center was established by the regioselective ring opening of a synthetic precursor (Baldwin et al., 1973). Kluender et al. (1973) confirmed these results by the stereoselective trans addition of hydrogen across the double bond of a ^{13}C-substituted precursor.

In both cases the *S* configuration was associated with the upfield resonance (signal at δ 17.8) and the *R* configuration with the downfield resonance (signal at δ 18.7).

The stereoselective organic synthesis of leucine has not been reported. Biosynthetic [5-^{13}C]leucine derived from *Escherichia coli* showed a strong peak at δ 21.4 which is the upfield resonance of those which correspond to the δ-carbons (Sylvester & Stevens, 1979). This suggests that the biosynthetic process converting α-ketoisovalerate to α-isopropylmalate produces (2*S*,4*S*)-[5-^{13}C]leucine. This is not proof, however, and the work presented herein was designated for that reason. By the stereospecific conversion of leucine enriched in one of the diastereotopic methyl groups to valine which should be enriched only in the same methyl group, the configuration of the parent leucine could be correlated to chemical shift positions observed in the ^{13}C NMR spectrum.

Materials and Methods

Chemicals. Silver(II) picolinate was prepared by the method of Clarke et al. (1969). Chiral (2*S*)-[5-^{13}C]leucine (70 atom % ^{13}C) was isolated from *E. coli* strain CU888 as described by Sylvester & Stevens (1979). D-Amino acid oxidase (15 units/mg; hog kidney) was purchased from Boehringer-Mannheim Biochemical Co. Catalase (30 000 units/mg) and FAD (95%; Grade III) were from Sigma Chemical Co. All other chemicals were reagent grade.

Silver(II) Picolinate Oxidation. A modification of the method of Clarke et al. (1970) was used to convert leucine to isovaleric acid. (2*S*)-[5-^{13}C]Leucine (70 atom % ^{13}C, 18.9 mg, 0.144 mmol) and (2*S*)-leucine (172.0 mg, 1.313 mmol) were dissolved in 12 mL of water and placed in a reaction vessel. Three traps were connected in series with the reaction vessel such that nitrogen could be bubbled through the system. The traps contained respectively 0.5 M potassium phosphate (pH 7.0) and 0.15 N $KMnO_4$ (4 mL), 0.33 M potassium phosphate (pH 7.0) and 0.25 N $KMnO_4$ (4 mL), and 0.5 M potassium phosphate (pH 7.0) and 0.15 N $KMnO_4$ (2 mL). Silver(II) picolinate (1.16 g, 3.33 mmol) was added to the reaction vessel which was shaken continuously with rapid passage of nitrogen in a 40 °C water bath. The water bath temperature was slowly raised to 70 °C over a period of 1 h and maintained at 70 °C until the suspended solids in the reaction vessel became white. The contents of the three traps were then combined and allowed to stand for 1 h at room temperature. After acidification (pH 2.0; HCl), the solution was extracted with chloroform (4 × 10 mL), and the combined chloroform extracts were backwashed with water (1 × 3 mL). The volume of the chloroform solution was reduced to 1 mL and then quantitatively transferred to a small ampule. The ampule was placed in a 50 °C water bath, and an air stream was passed into the neck of the ampule to evaporate the residual chloroform. A liquid residue (123 mg) remained and was not purified further. In control experiments, this procedure gave 85–90% yields of isovaleric acid (titration).

Conversion of [4-^{13}C]Isovaleric Acid to (2RS)-[4-^{13}C]Valine. Anhydrous bromine (0.1 mL, 1.93 mmol) and PCl_3 (0.01 mL, 0.12 mmol) were added to the ampule containing the impure residue. The ampule was then sealed and heated to

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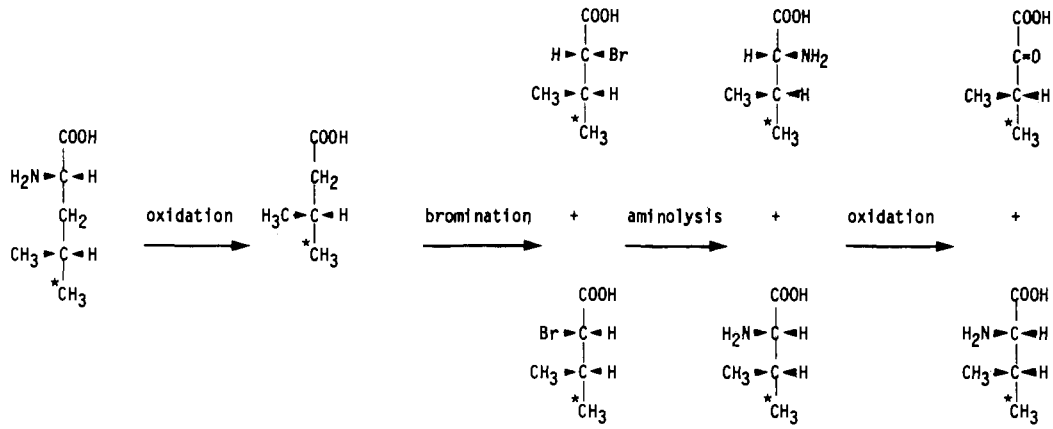
Scheme I: Conversion of Leucine to Valine



70 °C for 48 h in the dark. Excess bromine and HBr were allowed to evaporate from the opened ampule for 1 h at room temperature, leaving a crystalline mass of 2-bromoisovaleric acid.

Liquid NH_3 (1 mL) was added to the ampule which was sealed in a Parr bottle containing excess NH_3 (20 mL) (Baldwin et al., 1973). After 7 days (2*RS*)-[4-^{13}C]valine (~7 atom % ^{13}C) was recovered in a 25% overall yield with no other amino acid contamination as determined by amino acid analysis.

Isolation of (2S)-[4-^{13}C]Valine. The racemic valine mixture was resolved by incubation with D-amino acid oxidase (0.1 unit/mg) in the presence of catalse and FAD according to the procedure of Boulanger and Osteux (1965). The (2*S*)-[4-^{13}C]valine remaining was purified by ion-exchange chromatography (Sylvester & Stevens, 1979).

^{13}C Spectra. Spectra were recorded at 22.63 MHz in the Fourier transform mode of operation with proton broad band decoupling on a Bruker WP-90 by using 10-mm sample tubes. Ten percent D_2O served as the lock signal, and chemical shifts were determined relative to tetramethylsilane by using *p*-dioxane as the internal standard.

Results and Discussion

In Vivo Conversion of Acetolactate to Leucine by E. coli Strain CU888. Leucine isolated from *E. coli* strain CU888 grown in minimal media supplemented with L-isoleucine and (*RS*)-[2-$^{13}CH_3$]acetolactate gave a single strong peak in the ^{13}C NMR spectrum (δ 21.4). Another minor peak at δ 22.5 was observed and could be due to slight isomerization [cf. Armstrong et al. (1974)]. In another experiment, [^{14}C]leucine isolated from cultures grown in the presence of (*RS*)-[2-$^{14}CH_3$]acetolactate had the same specific activity as the $^{14}CH_3I$ used in the synthesis of labeled acetolactate. Thus, it can be concluded that all the leucine synthesized by *E. coli* strain CU888 was derived from acetolactate and that stereospecificity was maintained throughout the biosynthesis of leucine at the chiral center.

In Vitro Conversion of Leucine to Valine. The 70 atom % (2*S*)-[5-^{13}C]leucine derived from several cultures was pooled and combined with (2*S*)-leucine to give a workable quantity and yet allow enriched resonances to be easily discerned over natural abundance spectra. Potassium isovalerate derived after silver picolinate oxidation of (2*S*)-[5-^{13}C]leucine showed one strong peak (δ 22.8), demonstrating that configuration had been maintained. Bromination and aminolysis occurring at the α position of isovalerate would lead to (2*RS*)-[4-^{13}C]valine (Scheme I) but should not effect the stereochemistry at the β center [cf. Baldwin et al. (1973)]. The ^{13}C NMR spectrum of the synthesized (2*RS*)-[4-^{13}C]valine showed two strong peaks (δ 17.8 and δ 18.7) of nearly equal intensity. Upon incubation with D-amino acid oxidase and catalase, the peak at δ 18.7 diminished while a new peak at δ 17.4 became apparent ([4-^{13}C]-α-ketoisovalerate). The peak at δ 17.8 was uneffected. Purification over Dowex 50-X8 gave (2*S*,3*S*)-[4-^{13}C]valine with the ^{13}C NMR spectra showing δ 17.8 as the remaining enriched resonance.

Assignment of 2*S* configuraiton is by the stereospecificity of D-amino acid oxidase. Assignment of 3*S* configuration is based on documented ^{13}C NMR work [cf. Kluender et al. (1973) and Baldwin et al. (1973)]. Thus, by knowing the configuration of derived valine, one may obtain the absolute configuration of (2*S*,4*S*)-[5-^{13}C]leucine (Scheme I). This assignment agrees with the prediction that the *S* configuration at the chiral γ center is maintained because no structural changes should occur in the isopropyl group during biosynthesis of leucine from α-ketoisovalerate. The configurations of [4-^{13}C]isovalerate can be assigned as 3*S*, and this may be of value to future catabolic studies.

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Identification of 3-Methoxy-4-hydroxy-5-hexaprenylbenzoic Acid as a New Intermediate in Ubiquinone Biosynthesis by *Saccharomyces cerevisiae*†

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ABSTRACT: A ubiquinone-deficient mutant strain of *Saccharomyces cerevisiae*, 26H, was found to accumulate a previously unidentified intermediate in ubiquinone biosynthesis when grown in the presence of *p*-hydroxy[7-^{14}C]- or -[U-^{14}C]benzoic acid. This intermediate was isolated from the lipid extracts of a 100-L culture of 26H and purified by various chromatographic procedures to yield 20 mg of product. Analysis by means of NMR, IR, UV, and mass spectrometry revealed the structure of this new intermediate to be 3-methoxy-4-hydroxy-5-hexaprenylbenzoic acid (3-MHHB). In vitro experiments with isolated yeast and rat mitochondria showed that 3-MHHB could be converted to ubiquinone-6. These findings indicate that 3-O-methylation precedes decarboxylation of the prenylated protocatechuic acid intermediate in the biosynthesis of ubiquinone in eukaryotes.

The pathway for the biosynthesis of the electron-transport component ubiquinone has been described for prokaryotes by using mutants of *Escherichia coli* (Gibson & Young, 1978). For eukaryotes, however, the pathway from 4-hydroxy-5-polyprenylbenzoate to 6-methoxy-2-polyprenylphenol is thought to be different from that of prokaryotes. The compound 3,4-dihydroxy-5-hexaprenylbenzoic acid has been identified as an intermediate in the mitochondrial biosynthesis of ubiquinone by the lower eukaryote, *Saccharomyces cerevisiae* (Goewert et al., 1978). This intermediate was found to accumulate in methionine auxotrophs deprived of methionine, suggesting that methylation of the protocatechuate intermediate via *S*-adenosylmethionine, was the next step in the reaction sequence (Goewert et al., 1981). Additional support for this pathway comes from the work of Nambudiri et al. (1977), who found that mitochondrial preparations from rat heart and liver were able to prenylate both 3,4-dihydroxybenzoic acid and 3-methoxy-4-hydroxybenzoic acid. Other investigators (Casey & Threlfall, 1978) have shown that yeast mitochondria, when incubated with the ubiquinone precursors 4-hydroxybenzoate and isopentenypyrophosphate, or 3-hexaprenyl-4-hydroxybenzoate, synthesize 6-methoxy-2-hexaprenylphenol, 5-demethoxyubiquinone-6, and ubiquinone-6. Unfortunately the more labile carboxylated intermediates were not detected under their experimental conditions.

In this paper we report the identification of 3-methoxy-4-hydroxy-5-hexaprenylbenzoate, a new intermediate in the biosynthesis of ubiquinone-6. This compound was found to accumulate in a strain of *Saccharomyces cerevisiae* unable to synthesize ubiquinone-6. A preliminary report of this work has been made (Goewert et al., 1978). In addition, we have found that both yeast and rat mitochondria readily convert this compound to ubiquinone, demonstrating that the compound is the main pathway for ubiquinone biosynthesis in both higher and lower eukaryotes.

Materials and Methods

Male albino rats of either the Sprague-Dawley or Wistar strains, weighing 150 g and fed a stock commercial diet (Purina Laboratory Chow, Ralston Purina Co., St. Louis, MO) ad libitum, were used in these studies.

All chemicals were at least reagent grade: aluminum oxide from Aleysharm Chemicals and both analytical (0.25 mM) and preparative (2 mm) silica gel-60 thin-layer chromatography plates from Brinkmann, and cytochrome *c*, glutathione (reduced), dithiothreitol, 3-methoxy-4-hydroxybenzoic acid, and vanillic acid from Sigma. Ubiquinone standards and solanesol were the generous gifts of Dr. O. Wiss of Hoffmann-La Roche Ltd. (Basel, Switzerland). All ubiquinone pathways intermediates were synthesized following the procedure described by Nowicki et al. (1972). [*methyl*-^{3}H]-Methionine was purchased from New England Nuclear. *p*-Hydroxy[G-^{3}H]benzoic acid (800 mCi/mmol) was obtained from Amersham-Searle by custom synthesis under the direction of Dr. Bryan W. Baker. This product was purified to constant specific radioactivity by thin-layer chromatography (Nowicki et al., 1972). *p*-Hydroxy[U-^{14}C]benzoate was synthesized by sodium fusion of [U-^{14}C]tyrosine according to the method of Ho et al. (1973). *p*-Hydroxy[7-^{14}C]benzoate was prepared by catalytic reduction of *p*-benzyloxy[*carbox*-

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